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CYCLODEXTRIN GLYCOSYLTRANSFERASES FROM *Klebsiella pneumoniae* M 5 al AND *Bacillus macerans:* QUANTITATIVE ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE (1→4)-α-D-GLUCOPYRANOSYL TRANSFER-PRODUCTS FROM SOME LINEAR AND CYCLIC SUBSTRATES

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ABSTRACT

The analysis of the (1→4)-α-D-glucopyranosyl transfer-products from some linear and cyclic substrates by quantitative h.p.l.c. illuminated the mode of action of the cyclodextrin glycosyltransferases {(1→4)-α-D-glucan:[(1→4)-α-D-glucopyranosyl]transferase (cyclising), EC 2.4.1.19} from *Klebsiella pneumoniae* M 5 al and *Bacillus macerans*. D-Glucopyranosyl transfer, obligatory for maltose (poor substrate), was preferred for maltotriose (good substrate). The lengths of linear disproportionation-products increased with the lengths of the linear substrates. Cyclodextrins were produced from maltotriose and maltopentaose, but not from maltose. The cyclodextrins were substrates in the absence of acceptors. The cyclodextrin transformation started without the formation of detectable amounts of linear transfer-products. The cyclodextrin composition of long-term digests was nearly the same with all the cyclic substrates, cycloheptaamylose being the main cyclic compound. The linear carbohydrate was uniformly distributed from maltose up to at least maltononaose. The enzyme from *Bacillus macerans* was the least active, but long-term digests yielded results comparable to those obtained with the enzyme from *Klebsiella pneumoniae* M 5 al.

INTRODUCTION

Although interest in the cyclodextrins has increased during the last two decades[1–3], precise knowledge of the reaction mechanisms and the protein chemistry of the cyclodextrin glycosyltransferases {CGT, (1→4)-α-D-glucan:[(1→4)-α-D-glucopyranosyl]transferase (cyclising), EC 2.4.1.19} was hitherto incomplete. The reversible (1→4)-α-D-glucopyranosyl transfer-reactions characteristic of these enzymes, involving cyclisation, coupling, and disproportionation, are well known[4–11]. As shown for the CGT from *Klebsiella pneumoniae* M 5 al, maltosaccharides having chain lengths of 16–80 D-glucopyranosyl residues are necessary for

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maximum cyclisation rates, indicating a dependence on the helical conformation of the substrate[4-6]. α-D-Glucopyranose, maltosaccharides, α- or β-D-glucopyranosides, and some other sugars can serve as acceptors for the coupling reaction. Once formed, the linear chains are homologised. Chains of sufficient length are substrates for cyclisation. Cyclisation and disproportionation occur simultaneously with all the substrates assayed, confirming the close relationship between the reactions[4-6].

Detailed study of the reaction mechanisms has been hampered by a lack of suitable assay methods. We now describe the quantitative analysis by h.p.l.c. of the transfer products formed from some linear and cyclic substrates by CGT from *Klebsiella pneumoniae* M 5 al and from *Bacillus macerans*.

Materials. — CGT from *Klebsiella pneumoniae* M 5 al was isolated from the culture filtrate of continuously grown bacteria, and purified as previously described[12-14]. CGT from *Bacillus macerans* was a gift from Professor Szejtli (Budapest, Hungary). Glucoamylase [(1→4),(1→6)-α-D-glucan:glucohydrolase, EC 3.2.1.3; *Aspergillus niger*; 14 U/mg of protein] and the D-glucose test-kit GOD-Perid were obtained from Boehringer. Maltose (for biochemical purposes) was purchased from Merck, maltotriose was prepared[15,16] from pullulan digested with pullulanase [pullulan:(1→6)-glucanohydrolase, EC 3.2.1.41], and maltopentaose from cyclohexaamylose–maltose digests with CGT[4,5]. The maltosaccharides were purified by preparative h.p.l.c. The cyclodextrins were isolated from starch digests with CGT, and purified as described[17]. All other substances were commercial materials of the highest purity available.

Analytical methods — Total carbohydrates were determined with anthrone[18,19], and reducing aldehyde groups with the Nelson reagent[20]. Average chain-lengths were calculated[21] as the ratios of total carbohydrate to the reducing carbohydrate, both in glucose equivalents. Protein was determined by the biuret method[22], and glucose with D-glucose oxidase[23].

H.p.l.c. was performed on Waters μBondapak-NH_2 columns (3.9 × 300 mm), using acetonitrile–water (65:35) at 1.5 mL/min (1,200 p.s.i., 25°), with refractometric detection; 20 μL of each digest was injected. The carbohydrate contents of the elution peaks were calculated by planimetry, and calibrated with G_1–G_5 (glucose–maltopentaose) and cG_6–cG_8 (cyclohexa-, cyclohepta-, and cycloocta-amylose). The h.p.l.c. method allowed the detection[24] of chains up to G_{14}, and cG_6–cG_8 were eluted together with G_4–G_6. The amounts of G_4, G_5, and G_6 were calculated from those of the neighbouring shorter and longer chains. The cyclodextrin digests contained equal amounts of G_2, G_3, and G_7–G_9. It could be expected, therefore, that G_4–G_6 would be of the same order. For control purposes, the carbohydrates of the h.p.l.c. peaks of cG_6–G_4 and cG_7–G_5 were collected, and, after evaporation of the acetonitrile and addition of 50 μL of 20mM acetate buffer (pH 5.4), they were digested with glucoamylase (cG_6 and cG_7 are not substrates for the fungal enzyme[25]). The resulting glucose values agreed well with those calculated for the non-cyclic compounds from the h.p.l.c. data. Accordingly, the amounts of

cyclodextrins could be calculated from the differences of total peak carbohydrate and the linear-chain carbohydrate. The digests of G_2–G_5 contained decreasing amounts of chains G_2–G_{12}. A plot of the area of carbohydrate peaks *versus* chain lengths indicated the cyclodextrins eluted together with G_4–G_6.

Performance of the digests. — Each 1-mL sample of the substrate solution in 10mM Tris–HCl buffer (pH 7.2 for the CGT from *Klebsiella pneumoniae* M 5 al) or in 10mM acetate buffer (pH 5.4, for the CGT from *Bacillus macerans*), containing 5mM $CaCl_2$, was incubated with 50 μg (for the CGT from *Klebsiella pneumoniae*) and 80 μg (for the CGT from *Bacillus macerans*) of protein at 30° for 40 h. The initial substrate concentrations (water-free carbohydrate) were 90 (G_2), 60 (G_3), 30 (G_5), and 25mM (cG_6–cG_8). For h.p.l.c. analysis, 20 μL of the digests were withdrawn at intervals.

RESULTS AND DISCUSSION

The specific activities of the CGTs from *Klebsiella pneumoniae* M 5 al and *Bacillus macerans*, determined by the Kitahata method[26] and by the optical assay for the initial cyclisation rate[27], are summarised in Table I. The enzyme from *Bacillus macerans* was the least active. Inhibition by iodine[28] was unlikely, because the (1→4)-α-D-glucopyranosyl chains (lower activity) contained less iodine than the glycogen (higher activity). As calculated from published data[14,29,30], the molar catalytic activities (cyclisation reaction, starch, 30°) are 247 kat/mol for the CGT from *Bacillus macerans* (mol. wt. 145,000, dimeric), and 183.6 kat/mol for the CGT from *Klebsiella pneumoniae* M 5 al (mol. wt. 68,000, monomeric). Accordingly, the *Bacillus macerans* enzyme used for the present studies[31] differed from those de-

TABLE I

SPECIFIC ACTIVITIES OF THE CGT FROM *Klebsiella pneumoniae* M 5 al AND FROM *Bacillus macerans* DETERMINED BY THE KITAHATA METHOD[26], AND THE OPTICAL ASSAY FOR THE INITIAL CYCLISATION RATE[27a]

Assay	*CGT from*	
	K. pneumoniae *(U/mg of protein)*	B. macerans
Kitahata	1611	560 (34.7[b])
Initial cyclisation rate		
Glycogen	126	32 8 (26.3)
(1→4)-α-D-Glucopyranosyl chains (chain length, 16)	260	24.7 (9.5)

[a]The assays were performed in Tris–HCl buffer (pH 7.2, *Klebsiella pneumoniae* CGT), and in acetate buffer (pH 5.4, *Bacillus macerans* CGT), both containing 5mM $CaCl_2$. [b]Percent of activity determined for the *Klebsiella pneumoniae* CGT.

scribed by other researchers. In addition, it did not cause cyclisation at pH >6.5. Using sufficiently high concentrations of enzyme and pH 5.4, the composition of the mixture of transfer products was similar to that obtained with the CGT from *Klebsiella pneumoniae*.

Transfer products from linear substrates — Maltose (G_2) was a poor substrate for the CGTs. Since it contains only one (1→4)-α-D linkage, only D-glucopyranosyl groups can be transferred ($2G_2 \rightarrow G_3 + G_1$); >50% of substrate was present as D-glucose after incubation for 30 h with the CGT from *Klebsiella pneumoniae* M 5 al or *Bacillus macerans* (Fig. 1B,C). The amounts of linear disproportionation-products decreased rapidly with increase in chain length (Fig. 2A); the high concentrations of D-glucose and smaller maltosaccharides prevented the formation of chains $>G_5$, and, hence, cyclodextrins were not produced.

Maltotriose (G_3) was a good substrate for each CGT. From this sugar, maltosyl ($2G_3 \rightarrow G_5 + G_1$) or D-glucopyranosyl groups ($2G_3 \rightarrow G_4 + G_2$) can be transferred. Only traces of D-glucose and larger amounts of maltose were formed after incubation for 3 h, indicating that D-glucopyranosyl transfer was preferred (Fig. 3C). Presumably, the substrate binding-site of the enzyme is more specific for the maltosyl group than for the D-glucopyranosyl group, which would explain why maltose is a poor substrate but an excellent acceptor[6]. During short-term incubation, larger amounts of maltohexaose and maltooctaose could be detected by h.p.l.c. Prolonged incubation yielded higher amounts of D-glucose, and chains at least up to G_{11} (Figs. 2B and 3D). Due to the longer disproportionation-products, ~60%

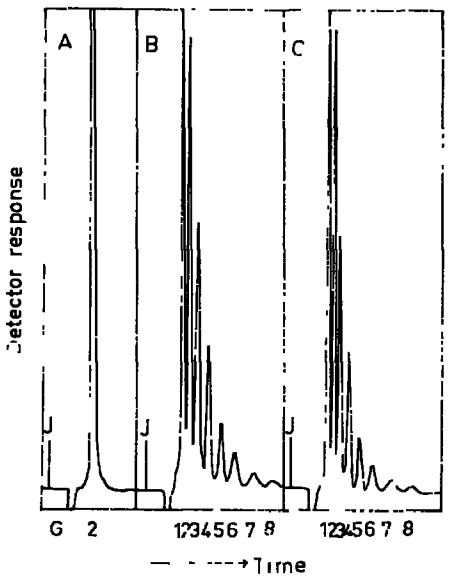

Fig. 1. H.p.l.c. of the transfer products after incubation (30 h, 30°) of G_2 (A) with CGT from *Klebsiella pneumoniae* M 5 al (B) and *Bacillus macerans* (C). 20 µl of each digest was injected (see Experimental).

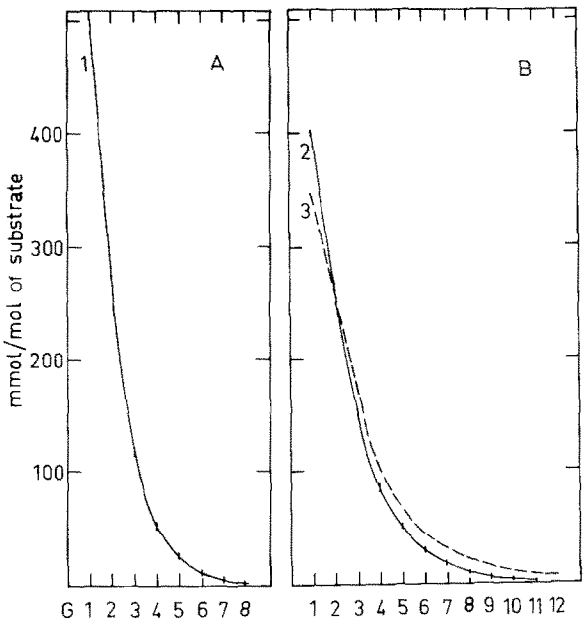

Fig. 2 Molar distribution of linear chains after incubation of G_2 (30 h, A1), G_3 (20 h, B2), and G_5 (20 h, B3) with the CGT from *Klebsiella pneumoniae* M 5 al and *Bacillus macerans* See Experimental for the calculation of the chain concentrations.

TABLE II

YIELDS OF CYCLIC TRANSFER-PRODUCTS FROM SOME LINEAR AND CYCLIC SUBSTRATES BY THE CGT FROM *Klebsiella pneumoniae* M 5 al[a]

Substrate[b]	*Time (30°) (h)*	*Total carbohydrate (%)*[c]	*cG_6,cG_7,cG_8 (mmol/mol of substrate)*[c]	*Molar ratio cG_6,cG_7,cG_8*
G_3	20	7.6	19 3,14.4,—	—
G_5	20	31	110,97,17	1:0.88:0.15
cG_6	15	75.9	205,388,77	1·1.89:0 37
cG_6	30	40.5	144,184,31	1:1 3:0.22
cG_7	40	58.7	186,344,48	1.1 85·0.26
cG_8	40	63 5	229,407,76	1:1.8:0.33
cG_6 + G_2[d]	20	41.2	140,199,36	1.1.42:0.26
cG_7 + G_2	30	41	136,256,30	1:1 88:0.22
cG_8 + G_2	20	48 4	140,263,38	1:1.88:0.27

[a]The digests were performed in 10mM Tris-HCl buffer (pH 7.2, containing 5mM $CaCl_2$). The concentration of the CGT was 50 μg of protein/mL. [b]The initial concentrations of substrate were 60 (G_3), 30 (G_5), and 25mM (cyclodextrins). [c]The amounts of cyclic compounds were determined by quantitative h.p.l.c [d]The acceptor concentration was 10mM.

TABLE III

YIELDS OF CYCLIC TRANSFER-PRODUCTS FROM G_3 AND THE CYCLODEXTRINS BY THE CGT FROM *Bacillus macerans*[a]

Substrate[b]	*Time (30°) (h)*	*Total carbohydrate (%)*[c]	*cG_6,cG_7,cG_8 (mmol/mol of substrate)*[c]	*Molar ratio cG_6:cG_7:cG_8*
G_3	30	8.3	25.4,12.3,—	—
cG_6	40	45.3	137,214,34	1:1.57:0.25
cG_7	40	53.9	173,308,43	1:1.78:0.25
cG_8	40	50.6	204,293.51	1:1.44:0.25

[a]The digests were performed in 10mM acetate buffer (pH 5.4, containing 5mM $CaCl_2$). The concentration of the CGT was 80 μg of protein/mL. [b]The initial concentrations of substrate were 60 (G_3) and 25mM (cyclodextrins). [c]The amounts of cyclic compounds were determined by quantitative h.p.l.c.

(*Klebsiella pneumoniae* CGT) and 8.26% (*Bacillus macerans* CGT) of the substrate was cyclised (Tables II and III).

Because of the larger size of the substrate, and the higher probability of longer disproportionation-products, more cyclodextrins should be produced with maltopentaose (G_5). Indeed, 31% of the substrate was cyclised after incubation for

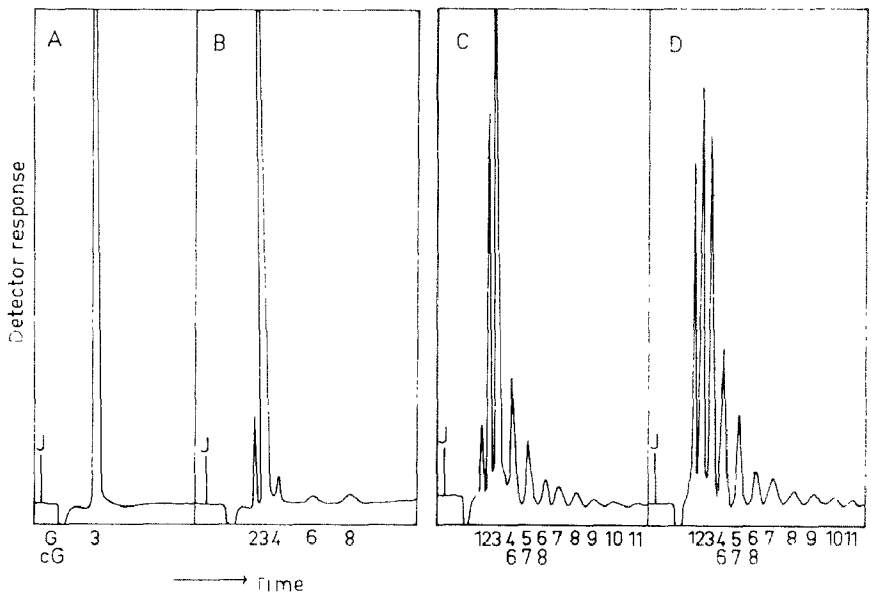

Fig. 3. H.p.l.c. of the transfer products obtained after incubation (30°) for 20 min (B), 3 h (C), and 20 h (D) of G_3 (A) with the CGT from *Klebsiella pneumoniae* M 5 a1. 20 μL of each digest was injected (see Experimental).

20 h, and the amounts of longer chains (up to at least G_{12}) were larger than those formed with the smaller substrates (Table II, Fig. 2B).

Transfer products from cyclodextrins. — Although the coupling reaction with cyclohexaamylose (cG_6) and suitable acceptors was studied[8], it is questionable whether the cyclodextrins are attacked in the absence of acceptors[32]. They proved to be substrates for the CGT from *Klebsiella pneumoniae* M 5 al and *Bacillus macerans* when sufficiently high concentrations of enzyme were used. The susceptibility to enzymic attack decreased in the order $cG_6 < cG_8 < cG_7$.

Coupling reactions with excess of the substrate cG_6 and very low concentrations of acceptor yielded chains long enough to stain blue with iodine, and to retrograde from solution[8]. The amounts of such long chains decrease with increase in enzyme concentration[17]. Accordingly, the enzyme:substrate ratio determines whether or not amylose-like chains are formed. Using high concentrations of enzyme, retrograding material could not be detected in the cyclodextrin digests. The transfer products shown by h.p.l.c. amounted to 90–95% of total carbohydrate (Figs. 4–6, Tables II and III).

Part of cG_6, for example, can be transformed into cG_7 during coupling and successive disproportionation reactions[33]. This transformation requires, by disproportionation, the synthesis of chains long enough for cyclisation. However, disproportionation yields a broad spectrum of chains having different lengths. As shown for cG_8 and cG_7, the cyclodextrin transformation started without the formation of detectable amounts of linear chains (Figs. 4B and 5B). Even after incubation for 20 h, the concentrations of maltosaccharides were very low (Fig. 4C).

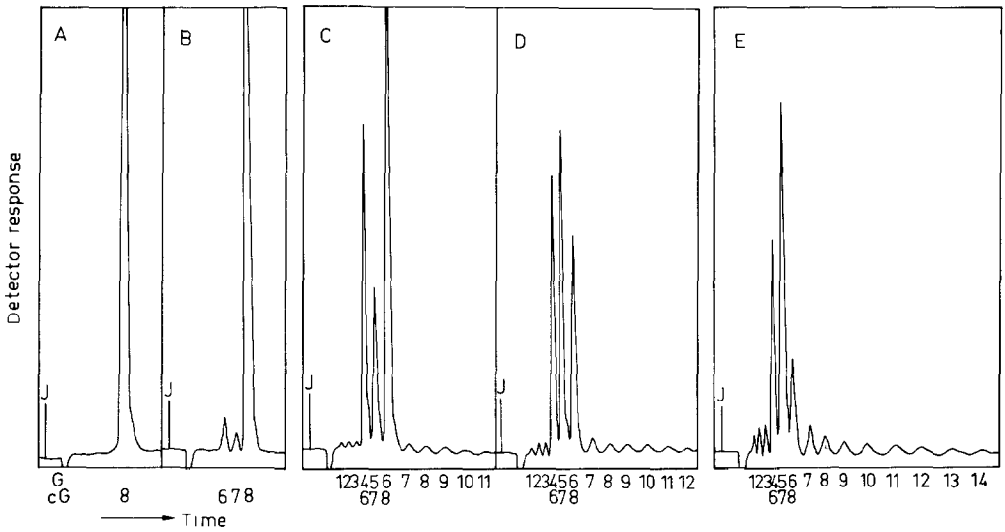

Fig. 4. H.p.l.c. of the transfer products obtained after incubation (30°) for 3 h (B), 20 h (C), 30 h (D), and 40 H (E) of cG_8 (A) with the CGT from *Klebsiella pneumoniae* M 5 al; 20 μL of each digest was injected (see Experimental).

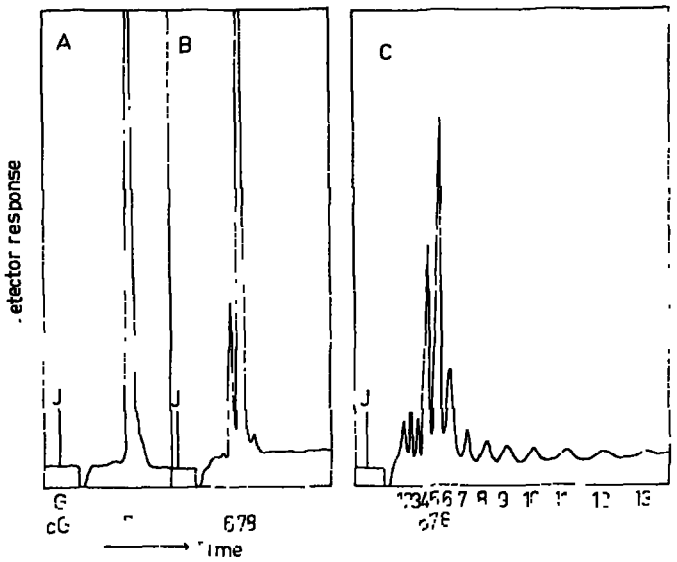

Fig 5 H p l c of the transfer products obtained after incubation (30°) for 20 h (B) and 40 h (C) of cG-(A) with the CGT from *Klebsiella pneumoniae* M 5 al. 20 μl of each digest was injected (see Experimental)

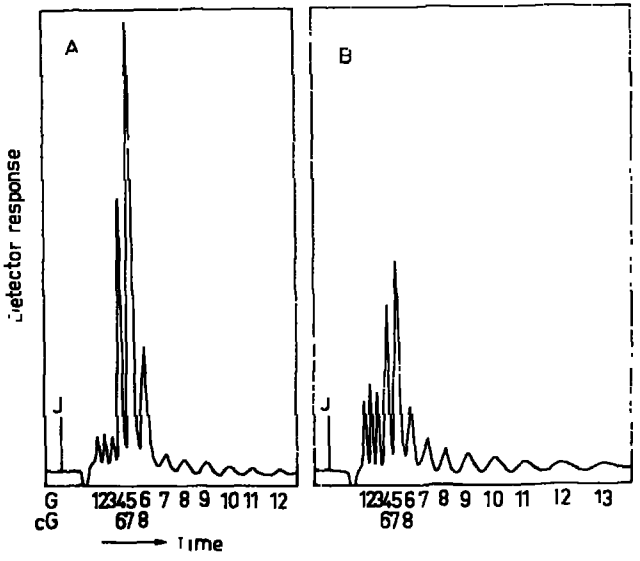

Fig 6 H.p l c of the transfer products obtained after incubation (30°) for 15 h (A) and 30 h (B) of cG_n with the CGT from *Klebsiella pneumoniae* M 5 al. 20 μl of each digest was injected (see Experimental)

Generally, (1→4)-α-D-glucopyranosyl transfer is initiated by the cleavage of a glucosidic bond in a chain at the substrate-binding site of the CGT. The part of the substrate containing the non-reducing end is transferred *via* HO-1 to HO-4 of an acceptor bound to the acceptor-binding site[4]. The cyclisation reaction is only a special type of this disproportionation, with the non-reducing chain end of one chain serving as the acceptor. Accordingly, the substrate for cyclisation must be bound to both the substrate- and the acceptor-binding sites of the enzyme. For the reverse reaction, a cyclodextrin molecule must be bound to the substrate-binding site, and the resulting linear chain may be transferred to the acceptor-binding site, thus serving as acceptor for a successive coupling-reaction. If, for example, cG_8 or cG_7 were present at the substrate-binding site and the resulting linear chain was transferred to the acceptor-binding site, coupling with another cG_8 or cG_7 would yield enzyme-bound G_{16} or G_{14}, which are substrates for cyclisation. Thus, the linear product must not be released from the enzyme.

The (1→4)-α-D-glucopyranosyl transfer-reactions catalysed by the CGT are reversible. Equilibrium constants for the cyclodextrins[33], however, cannot be determined precisely, because the concentration of chains long enough for cyclisation alters continually: 75.9% of total carbohydrate was present as cyclic compounds after incubation for 15 h with cG_6 (Fig. 6A, Table II). After incubation for 30 h, the amount of cyclodextrins decreased to 40.5% of the total carbohydrate at the expense of shorter chains (Fig. 6B, Table II). The composition of the mixture of transfer products was similar to that of the digests with the cyclodextrins plus added acceptor (Table II). Evidently, there was no defined reaction-equilibrium, because both coupling and disproportionation occurred. If there is an equilibrium of the

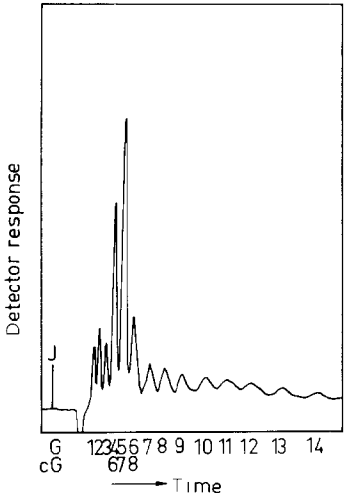

Fig. 7. H p.l.c. of the transfer products obtained after incubation (30°) for 40 h of cG_7 with the CGT from *Bacillus macerans*; 20 μL of the digest was injected (see Experimental). The composition of the digests with cG_6 and cG_8 was nearly identical.

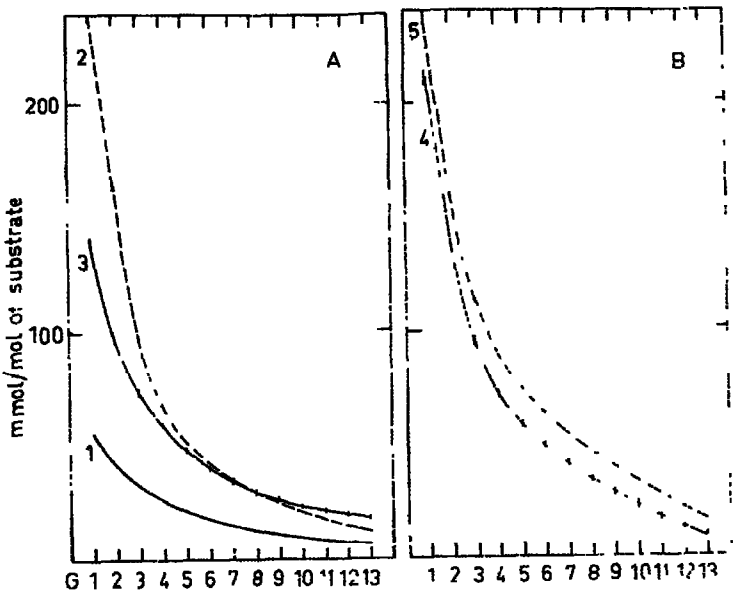

Fig. 8. Molar distribution of linear chains of the cyclodextrin digests with the CGT from *Klebsiella pneumoniae* M 5 al (A) and *Bacillus macerans* (B). Curves 1 and 2, cG_n [incubation (30°) for 15 h and 30 h, respectively], curve 3, cG_7 and cG_8 [incubation (30°) for 40 h, the chain composition was similar for both digests], curve 4, cG_6 and cG_7 [incubation (30°) for 40 h], curve 5, cG_6 [incubation (30°) for 40 h]. See Experimental for the calculation of the chain concentrations.

cyclisation reaction between chains $>G_{14}$ and the cyclodextrins, then the increase in shorter chains must influence the equilibrium.

Because of its lower susceptibility to enzymic attack, cG_7 was the main cyclodextrin formed from all the cyclic substrates (Tables II and III). Generally, the amounts of linear products increased with the time of incubation (Fig. 8A, curves 1 and 2); the average chain-length of the maltosaccharides was 3.8–4.3. The distribution of linear carbohydrates was uniform and, except for D-glucose, similar amounts (by weight) were obtained from maltose up to at least maltononaose.

The analysis of the long-term digests with the CGT from *Bacillus macerans* yielded similar results (Figs. 7 and 8B, Table III). No marked differences existed between the digests with cG_6, cG_7, and cG_8.

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